

The silencing of LncRNA-H19 decreases chemoresistance of human glioma cells to temozolomide by suppressing epithelial-mesenchymal transition via the Wnt/ β -Catenin pathway

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Introduction: Temozolomide (TMZ) is commonly used for glioma chemotherapy. However, TMZ resistance limits the therapeutic effect of TMZ in glioma treatment. LncRNA-H19 acts as an oncogenic LncRNA in some types of cancers and has been reported to be up-regulated in glioma.

Materials and methods: In our present study, we established TMZ-resistant glioma cells (U-251TMZ and M059JTMZ) to explore the effect of H19 on the chemoresistance of glioma cells.

Results: We observed that the expression of H19 was significantly increased in U-251TMZ and M059JTMZ cells. Knockdown of H19 expression using specific shRNA in U-251TMZ and M059JTMZ led to decreased half maximal inhibitory concentration (IC_{50}) values for TMZ and increased cell apoptosis rates, indicating that the silencing of H19 decreased chemoresistance of glioma cells to TMZ. In addition, silencing of H19 suppressed epithelial-mesenchymal transition (EMT) by increasing the expression of epithelial marker E-cadherin and decreasing the expression of mesenchymal marker Vimentin and ZEB1. Moreover, inducing EMT by TGF- β 1 treatment led to increased IC_{50} values for TMZ and decreased cell apoptosis rates compared with TMZ+H19 shRNA group, suggesting that the induction of EMT counteracted the inhibitory effect of H19 shRNA on chemoresistance of glioma cells to TMZ. Furthermore, the reduced expression of H19 down-regulated the expression of β -Catenin and its downstream targets c-myc and Survivin in TMZ-treated glioma cells. Activation of Wnt/ β -Catenin pathway by LiCl treatment promoted EMT and enhanced chemoresistance to TMZ compared with TMZ+H19 shRNA group.

Conclusion: Taken together, our data suggest that H19 decreased chemoresistance of glioma cells to TMZ by suppressing EMT via the inhibition of Wnt/ β -Catenin pathway. Our study might represent a novel therapeutic target for TMZ-resistant glioma.

Keywords: LncRNA-H19, chemoresistance, glioma, temozolomide, EMT, Wnt/ β -Catenin

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Introduction

Glioma is the most common and aggressive malignant brain tumor in the central nervous system with a high rate of recurrence and mortality.¹ Although standard treatments including surgical resection, radiation, and chemotherapy have been improved for glioma, the prognosis for patients with glioma is still very poor, with a median

survival of less than 15 months.² Several chemotherapeutic agents have been used for glioma treatment. Temozolomide (TMZ), one of the chemotherapeutic agents for glioma, is reported to improve the overall survival of glioma patients after concurrent postoperative use of TMZ.³ TMZ attacks the O(6) position on guanine, inducing DNA strand breaks of growing tumor cells.⁴ However, TMZ resistance is a serious impediment in the treatment of glioma.⁵ Hence, a better understanding of the mechanism related to TMZ resistance can help to improve the poor prognosis of glioma patients.

LncRNA is a class of RNA which consists of more than 200 nucleotides with no-protein-coding capacity.⁶ Recently, LncRNAs have been identified to be involved in a great number of important cellular processes including cell proliferation, cell apoptosis, migration, and invasion.⁷ Moreover, increasing evidence has indicated that abnormal expression of LncRNA was often observed in various tumors and contributed to tumor progression, invasion, and chemoresistance.^{8,9} LncRNA-H19 acts as an oncogenic LncRNA in some types of cancers such as breast cancer, hepatocellular carcinoma, and bladder cancer.^{10,11} As in glioma, the expression of H19 was up-regulated and promoted tumor progression by binding to transcription factor *c-myc*.¹² Li et al's study reported that suppressing the expression of H19 inhibited tumorigenicity and stemness in U251 and U87MG glioma cells.¹³ However, the relationship between H19 and the development of chemoresistance to TMZ is not well established in glioma cells.

Epithelial-mesenchymal transition (EMT), a cellular switch from epithelial to mesenchymal properties, is reported to reduce intercellular adhesion and promote cell migration in various cancers.^{14,15} Previous studies elucidated that EMT not only promoted cell migration but was also involved in chemoresistance of tumor cells. Wen et al's study indicated that EphA2 affected the sensitivity of oxaliplatin by inducing EMT in oxaliplatin-resistant gastric cancer cells.¹⁶ Gaianigo et al also reported the relationship between EMT and treatment resistance in pancreatic cancer.¹⁷ However, the interaction between EMT and chemoresistance of glioma cells to TMZ is still unclear and requires more investigation.

In our present study, we determined the expression level of H19 in TMZ-resistant glioma cells and explored the function and potential mechanism of H19 in the chemoresistance of glioma cells to TMZ. We found that H19 was highly expressed in TMZ-resistant glioma cells, and silencing of H19 decreased chemoresistance to TMZ by suppressing EMT via the Wnt/ β -Catenin pathway in glioma cells. Our study highlighted the novel connection between H19 and chemoresistance to TMZ, which could be targeted in glioma treatment.

Materials and methods

Cell culture

Human glioma cell lines U-251 and M059J were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution at 37°C in a humidified 5% CO₂ incubator. Stock solution of TMZ (Schering-Plough, Kenilworth, NJ, USA) was prepared by dissolving the drug in dimethyl sulfoxide (DMSO). TGF- β 1 (R&D Systems, Inc., Minneapolis, MN, USA), an EMT inducer, was used to treat cells at the concentration of 4 ng/mL in our study. LiCl (Sigma-Aldrich Co., St Louis, MO, USA), a Wnt/ β -Catenin pathway activator, was also used to treat cells at the concentration of 20 mM. U-251 and M059J cells were initially treated with 10 μ M TMZ for 2 weeks, and then the TMZ concentration was increased by approximately two-fold for every two passages until it reached 640 μ M (maximum concentration kills all resistant cells, and the established cells were induced at 200 μ M TMZ) to establish TMZ-resistant cell lines.¹⁸

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells using TRIzol Reagent (Life Technologies, Thermo Fisher Scientific) according to the standard protocol. RNA (1 μ g) was reverse-transcribed to cDNA using QuantiTect Reverse Transcription Kit (Qiagen NV, Venlo, the Netherlands) following the manufacturer's instructions. Power SYBR Green (Takara, Dalian, China) was used for detection of H19 expression. qRT-PCR was performed with an initial 2 min denaturation step at 95°C, 40 cycles of a 15 sec denaturation step at 95°C, followed by a 60 sec hybridization step at 60°C on the LightCycler 480 (Hoffman-La Roche Ltd., Basel, Switzerland). GAPDH was used as an endogenous control for H19 expression level. The relative level was calculated by the relative quantification ($2^{-\Delta\Delta C_t}$) method. The PCR primers for H19 and GAPDH were as follows: H19: sense: GGCTCTGGAAGCTAGA GGAA; anti-sense: CTGGGATGATGTGGTGGC; GAPDH: sense: TGTGGGCATCAATGGATTTGG; anti-sense: ACACCATGTATTCCGGGTCAAT.

Transfection

The shRNA directly against LncRNA H19 and negative control H19 scramble (GenePharma, Shanghai, China) were cloned into vectors respectively. Transfections were performed using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's instructions. qRT-PCR was performed to confirm the efficiency after transfection.

MTT assay

MTT assay was used to evaluate cell viability. Cells were seeded into 96-well plates at the concentration of 2×10^3 cells/well. Cells were treated with different concentrations of TMZ (0, 500, 1,000, 1,500, 2,000, 2,500 μM) for 24 h. Then, MTT was added to each well (10 μL , 10 mg/mL) and incubated in the dark at 37°C for 2 h. Absorbance was determined at a wavelength of 570 nm.

Cell apoptosis analysis

Cell apoptosis was detected by flow cytometric analysis. Annexin V-fluoresceine isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (Multisciences, Shanghai, China) was used for apoptosis analysis. Cells (2×10^5) were washed in PBS twice and resuspended in 500 μL annexin-binding buffer. The percentage of live cells was more than 90% before we began our experiments. Then, 5 μL of Annexin V-FITC and 10 μL of PI were added into the suspension for incubation. After incubating for 20 min in the dark and 4',6-diamidino-2-phenylindole (DAPI) staining, apoptotic cell death was analyzed by flow cytometry (Becton-Dickinson, Franklin Lakes, NJ, USA).

Western blot analysis

Proteins were lysed by radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) and the concentrations were measured using the Bio-Rad protein assay kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) according to the manufacturer's instructions. Proteins were separated by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories Inc., Hercules, CA, USA). Then, the membranes were blocked with 5% skim milk at room temperature for 1 h. After washing with tris-buffered saline tween-20 (TBST), the membranes were incubated with primary antibodies (β -Catenin, c-myc, Survivin, E-cadherin, Vimentin, ZEB1, and GAPDH antibodies purchased from Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C . After incubating with corresponding secondary antibodies (1:5,000; Cell Signaling Technology) at room temperature for 1 h, the ECL system (Bio-Rad Laboratories Inc.) was used for detection.

Statistical analysis

Experimental data are presented as mean \pm SD from three independent experiments using Student's *t*-test and/or one-way analysis of variance to perform appropriate data comparisons by SPSS 19.0. The difference was considered statistically significant at $P < 0.05$.

Results

H19 is up-regulated in TMZ-resistant glioma cells and silencing of H19 decreases chemoresistance of glioma cells to TMZ

We established TMZ-resistant glioma cell lines and measured the expression of H19 in the related cells. We found that the expression of H19 was significantly up-regulated in TMZ-resistant glioma cell lines U-251TMZ and M059JTMZ compared with the untreated U-251 and M059J cells, respectively (Figure 1A). H19 shRNA was transfected into TMZ-resistant glioma cells to decrease the expression of H19 and qRT-PCR was performed to confirm the efficiency after transfection (Figure 2B). Moreover, the the half maximal inhibitory concentration (IC_{50}) values of TMZ were approximately 600 μM and 1,000 μM in TMZ+H19 shRNA and TMZ+H19 scramble groups of U-251TMZ cells, respectively. The IC_{50} values of TMZ were approximately 400 μM and 800 μM in TMZ+H19 shRNA and TMZ+H19 scramble groups of M059JTMZ cells, respectively. These results suggested that H19 shRNA increased the sensitivity to TMZ of TMZ-resistant glioma cell lines (Figure 1C and D). In addition, silencing of H19 increased cell apoptosis rates of both U-251TMZ and M059JTMZ cells compared with TMZ+H19 scramble group in TMZ treatment (Figure 1E–H). Taken together, our data indicated that silencing of H19 decreases chemoresistance of glioma cells to TMZ.

Silencing of H19 inhibits EMT in glioma cells

We chose U-251TMZ cells for the following experiments because these cells were more resistant to TMZ. EMT was reported to be involved in chemoresistance of tumor cells. Thus, we detected the expression of EMT-related proteins through Western blot. Our data showed that the expression of epithelial marker E-cadherin was up-regulated while the expression of mesenchymal markers Vimentin and ZEB1 was down-regulated significantly by H19 shRNA compared with the H19 scramble group in TMZ treatment (Figure 2A–D). These results suggested that the down-regulation of H19 suppressed EMT in glioma cells.

Inducing EMT counteracts the inhibitory effect of H19 shRNA on chemoresistance of glioma cells to TMZ

In order to further explore the interaction between EMT and chemoresistance, TGF- β 1 was used to induce EMT in our study. Our results showed that the expression of E-cadherin was decreased while the expression of Vimentin

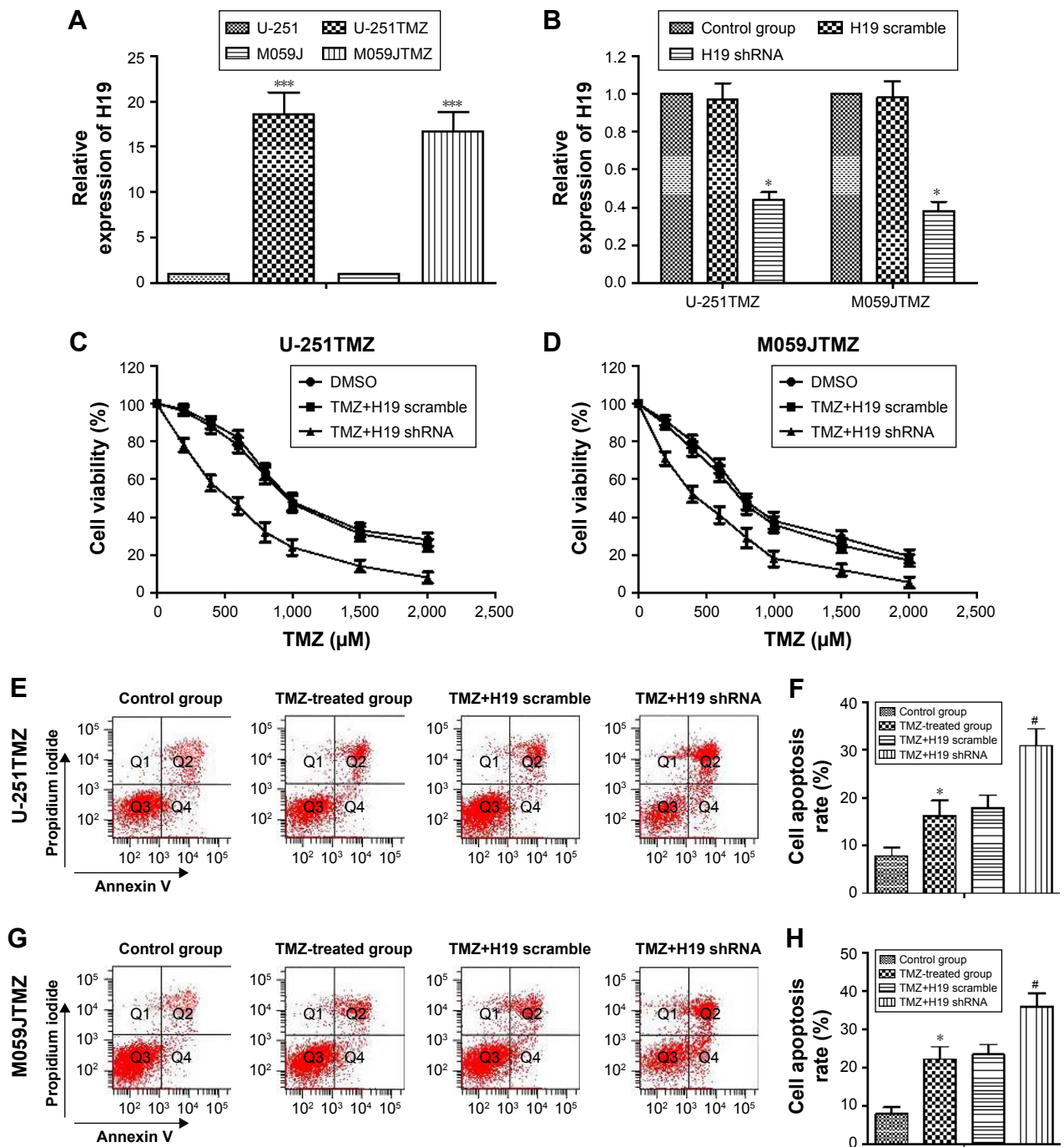


Figure 1 H19 is up-regulated in TMZ-resistant glioma cells and silencing of H19 decreases chemoresistance of glioma cells to TMZ.

Notes: (A) Relative expression of H19 was detected in glioma cells (U-251, M059J) and TMZ-resistant glioma cells (U-251TMZ, M059JTMZ) by qRT-PCR. $***P < 0.001$ compared with U-251 or M059J, respectively. (B) Relative expression of H19 was detected in U-251TMZ or M059JTMZ cells transfected with H19 scramble or H19 shRNA and in the control group, by qRT-PCR. $*P < 0.05$ compared with H19 scramble group. (C, D) Cell viability was measured by MTT assay. (E–H) Cell apoptosis rate was measured by flow cytometric analysis. The bars show mean \pm SD of three independent experiments. $*P < 0.05$ compared with control group, $#P < 0.05$ compared with TMZ+H19 scramble group.

Abbreviations: DMSO, dimethyl sulfoxide; qRT-PCR, quantitative real-time polymerase chain reaction; TMZ, temozolomide.

and ZEB1 was increased significantly by TGF- β 1 treatment compared with TMZ+H19 shRNA group (Figure 3A and B). Apart from that, the IC_{50} values of TMZ were approximately 400 μ M and 700 μ M in H19 shRNA group and H19 shRNA+TGF- β 1 group respectively, suggesting that

activation of EMT by TGF- β 1 treatment decreased the sensitivity to TMZ of TMZ-resistant glioma cells (Figure 3C). Meanwhile, cell apoptosis rate was decreased by TGF- β 1 compared with TMZ+H19 shRNA group (Figure 3D). Taken together, these results indicated that induced EMT weakened

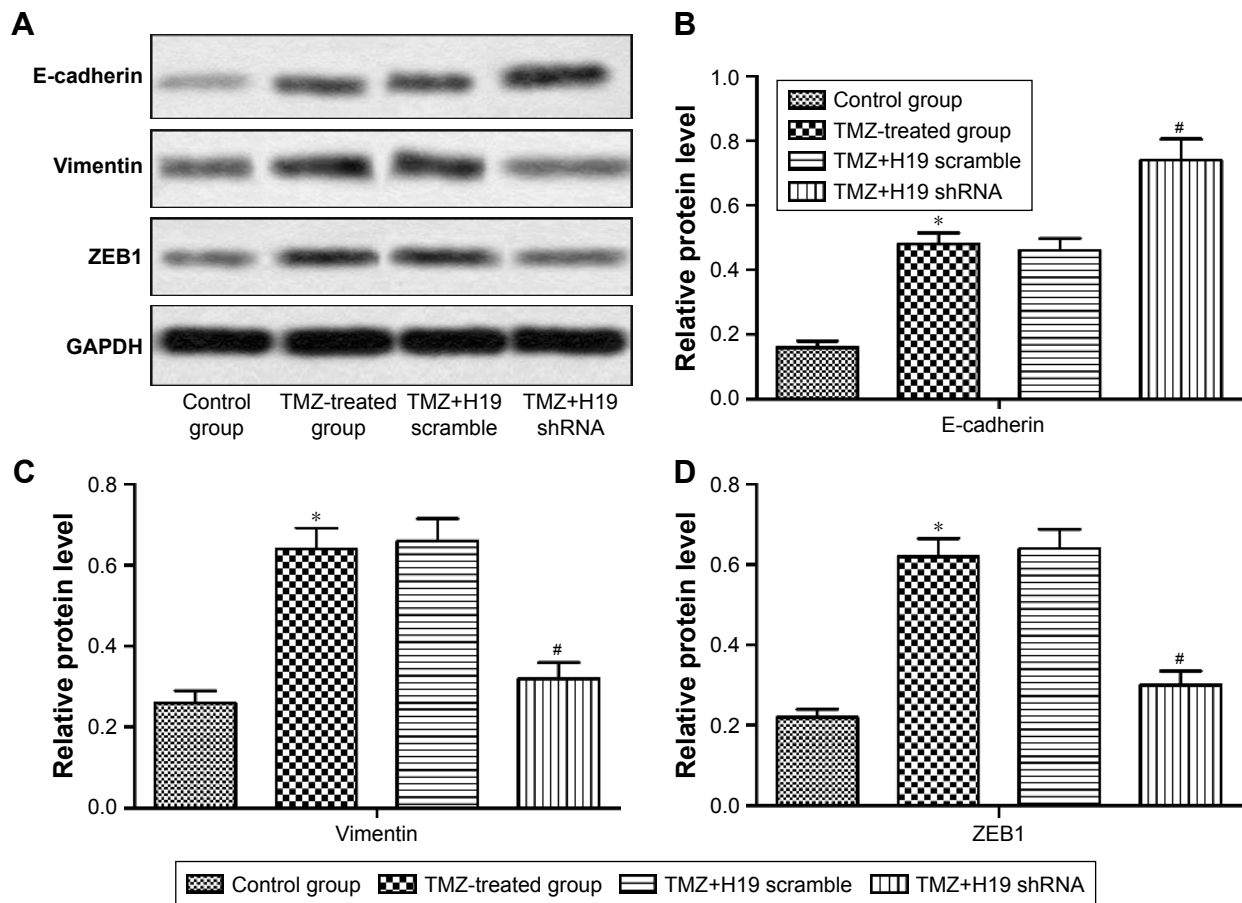


Figure 2 Silencing of H19 inhibits EMT in glioma cells.

Notes: U-251TMZ cells transfected with or without H19 scramble or H19 shRNA received TMZ treatment. (A–D) Relative protein level of E-cadherin, Vimentin, and ZEB1 was measured by Western blot with GAPDH as an endogenous reference. The bars show mean \pm SD of three independent experiments. * $P < 0.05$ compared with control group, # $P < 0.05$ compared with TMZ+H19 scramble group.

Abbreviations: EMT, epithelial-mesenchymal transition; TMZ, temozolomide.

the inhibitory effect of H19 shRNA on chemoresistance of glioma cells to TMZ.

Silencing of H19 decreases chemoresistance through suppressing EMT via the Wnt/ β -Catenin pathway

The Wnt/ β -Catenin pathway was reported to be involved in chemoresistance in some cancer cells.^{19,20} In our study, we detected the expression of β -Catenin, c-myc, and Survivin, which was related to Wnt/ β -Catenin pathway, through Western blot. Our data showed that the expressions of β -Catenin, c-myc, and Survivin were all down-regulated by H19 shRNA compared with TMZ+H19 scramble group (Figure 4A and B). However, LiCl, which acted as a Wnt/ β -Catenin pathway activator, increased the expression of β -Catenin compared with TMZ+H19 shRNA group (Figure 4C and D). Moreover, the expression of E-cadherin was decreased while the expression of Vimentin was increased significantly by LiCl treatment compared with

TMZ+H19 shRNA group, indicating that the activation of Wnt/ β -Catenin pathway promoted EMT in H19 shRNA transfected glioma cells (Figure 4E and F). Moreover, the IC_{50} values of TMZ were approximately 400 μ M and 700 μ M in H19 shRNA group and H19 shRNA+LiCl group respectively, suggesting that activation of Wnt/ β -Catenin pathway decreased the sensitivity to TMZ of TMZ-resistant glioma cells (Figure 4G). LiCl treatment decreased cell apoptosis rate compared with TMZ+H19 shRNA group (Figure 4H). Taken together, our data elucidated that the silencing of H19 decreased chemoresistance of glioma to TMZ by suppressing EMT via inhibiting the Wnt/ β -Catenin pathway.

Discussion

Glioma is an aggressive disease characterized by rapid progression, early metastasis, and marked resistance to chemotherapy. The survival rate is extremely low with median overall survival of 7–15 months.²¹ This aggressive disease is often treated with a combination of surgery,

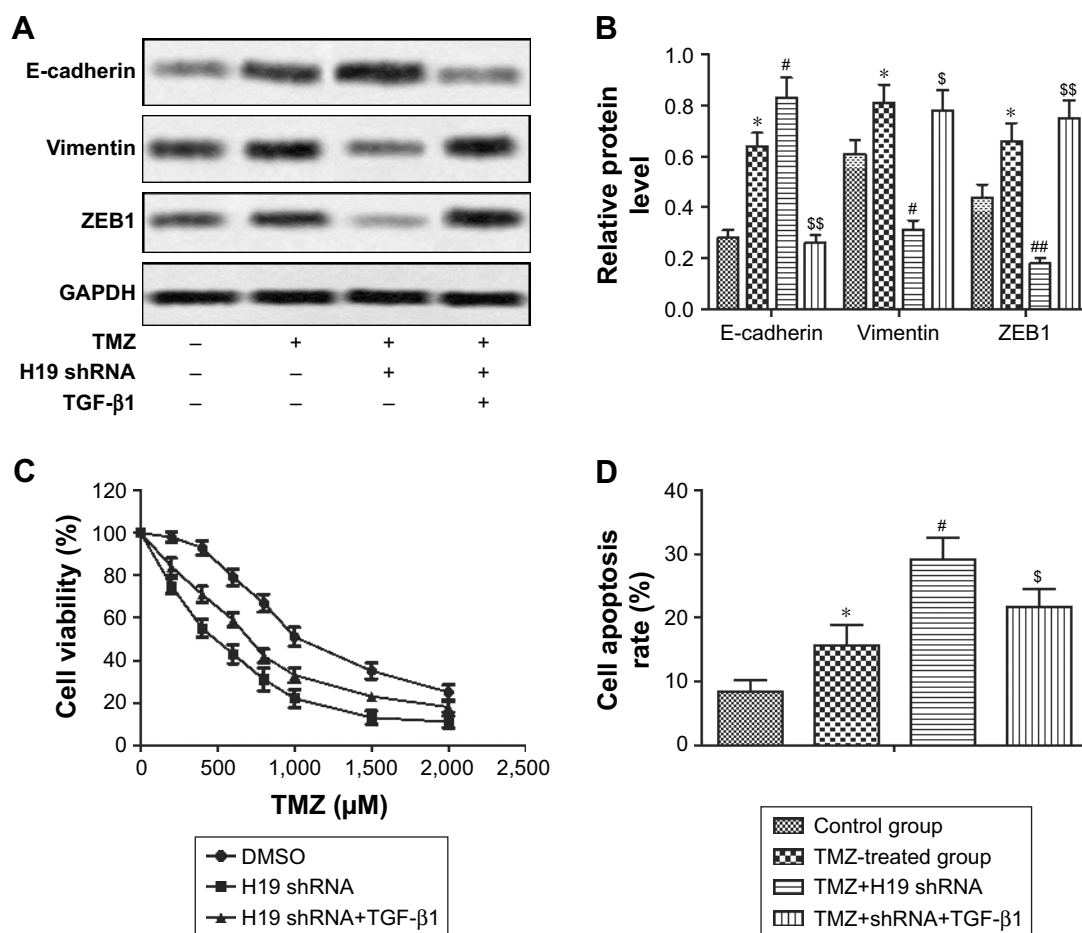


Figure 3 Inducing EMT counteracts the inhibitory effect of H19 shRNA on chemoresistance of glioma cells to TMZ.

Notes: U-251TMZ cells transfected with H19 shRNA were treated with TMZ combined with or without TGF-β1. (A, B) Relative protein level of E-cadherin, Vimentin, and ZEB1 was measured by Western blot with GAPDH as an endogenous reference. (C) Cell viability was measured by MTT assay. (D) Cell apoptosis rate was measured by flow cytometric analysis. The bars show mean ± SD of three independent experiments. * $P < 0.05$ compared with control group, # $P < 0.05$, ## $P < 0.01$ compared with TMZ-treated group, \$ $P < 0.05$, \$\$ $P < 0.01$ compared with TMZ+H19 shRNA group.

Abbreviations: DMSO, dimethyl sulfoxide; EMT, epithelial-mesenchymal transition; TMZ, temozolomide.

radiation, and chemotherapy to improve prognosis of patients.²² TMZ is usually used as a frontline chemotherapy agent to treat glioma and is reported to enter the cerebrospinal fluid without hepatic metabolism for activation.²³ However, accumulative TMZ resistance limits the therapeutic effect of TMZ in glioma treatment. Hence, strategies to overcome TMZ resistance are urgently needed. In our present study, we explored the possibility of LncRNA H19's involvement in chemoresistance of glioma cells to TMZ. Elevated expression of H19 was observed in TMZ-resistant glioma cell lines. Transfection of H19 shRNA decreased chemoresistance of glioma cells to TMZ by suppressing EMT via the Wnt/β-Catenin pathway, providing novel insights into the TMZ resistance in glioma.

Accumulated evidence indicates that LncRNA is a crucial regulator in the progression of glioma. Wu et al's study indicated that up-regulation of LncRNA HOXA-AS3

promoted tumor progression and acted as a poor prognosis marker in glioma.²⁴ Silencing of LncRNA-CCDC26 was reported to restrain the growth and migration of glioma cells.²⁵ Another LncRNA H19 had also gained attention of researchers recently. H19 is one of the most highly abundant and conserved transcripts in mammalian development and plays an important role in limiting growth of the placenta before birth.²⁶ H19 was reported to have an oncogenic role in various tumors. H19 was significantly up-regulated in some tumors such as gastric cancer and bladder cancer.^{27,28} Zhao et al elucidated that H19 was up-regulated in glioma cells and promoted glioma cell growth through targeting miR-140.²⁹ It was reported that H19 drove tumor transformation by binding with transcription factor c-myc and contributed to tumorigenesis.¹² However, the role of H19 in chemoresistance to TMZ has not been well studied previously. Our study revealed the crucial finding that H19 was highly expressed in

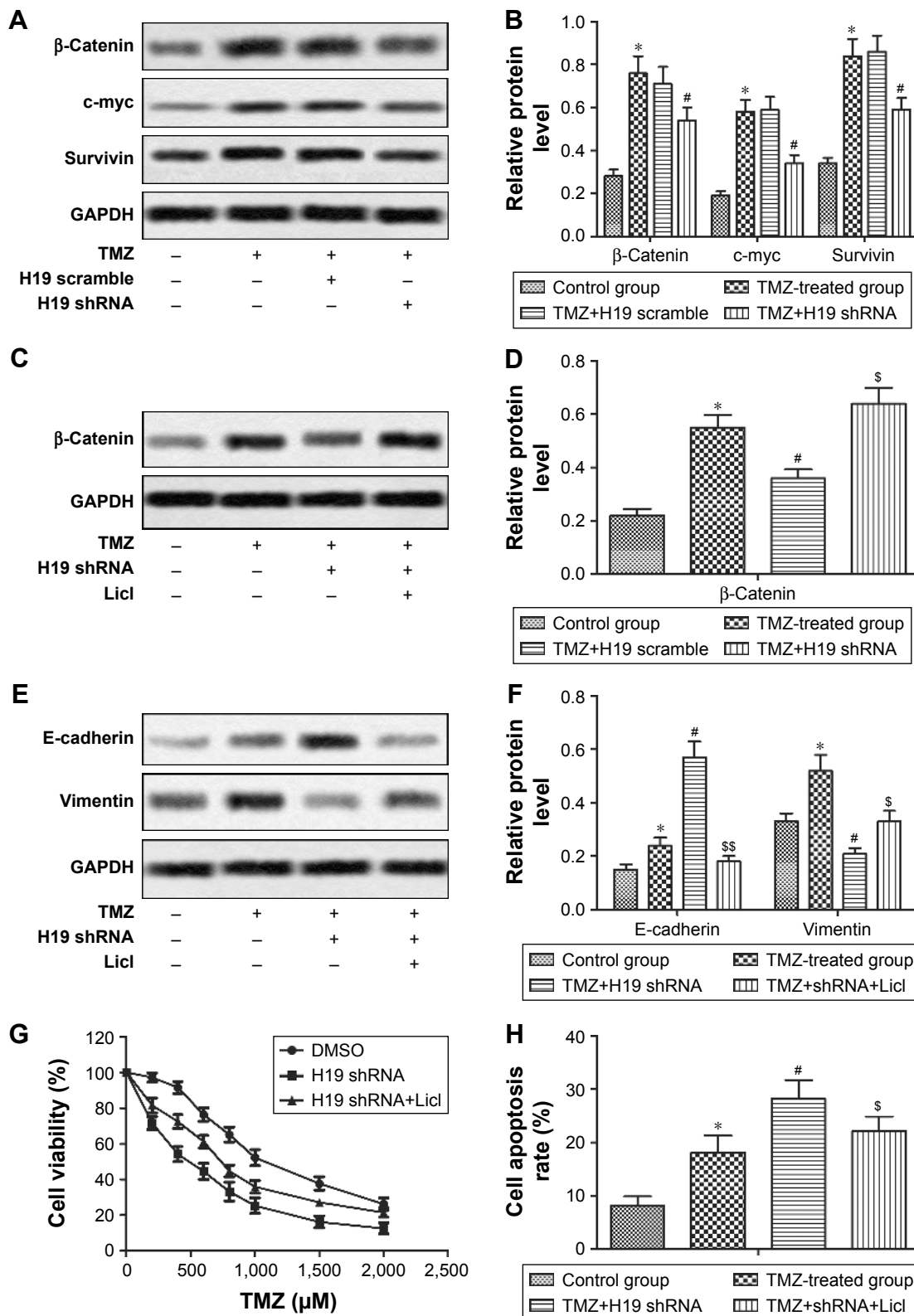


Figure 4 Silencing of H19 decreases chemoresistance through suppressing EMT via the Wnt/β-Catenin pathway. **Notes:** U-251TMZ cells transfected with H19 shRNA were treated with TMZ combined with or without LiCl. **(A, B)** Relative protein level of β-Catenin, c-myc, and Survivin was measured by Western blot with GAPDH as an endogenous reference. **(C-F)** Relative protein level of β-Catenin, E-cadherin, and Vimentin was measured by Western blot with GAPDH as an endogenous reference. **(G)** Cell viability was measured by MTT assay. **(H)** Cell apoptosis rate was measured by flow cytometric analysis. The bars show mean ± SD of three independent experiments. **P*<0.05 compared with control group, #*P*<0.05 compared with TMZ-treated group, \$*P*<0.05, \$\$*P*<0.01 compared with TMZ+H19 shRNA group.

Abbreviations: DMSO, dimethyl sulfoxide; EMT, epithelial-mesenchymal transition; TMZ, temozolomide.

TMZ-resistant glioma cells (U-251TMZ and M059JTMZ). Knockdown of H19 expression using specific shRNA in U-251TMZ and M059JTMZ led to decreased IC₅₀ values for TMZ and increased cell apoptosis rates, indicating that the silencing of H19 decreased chemoresistance of glioma cells to TMZ.

EMT is an important cellular process by which epithelial cells are transformed into mesenchymal cells.³⁰ A great number of studies have revealed that EMT is one of the key initiating events in the metastatic cascade and promotes tumor cells' metastasis.³¹ Nowadays, EMT has also been shown to be an important step in inducing drug resistance of tumor cells.³² Huang et al's research observed that cisplatin-resistant gastric cancer cells underwent a morphological change similar to EMT which was mediated by HER2 overexpression.³³ Previous studies suggested that H19 was related to EMT in tumor progression. A study by Zhao et al indicated that H19 promoted endometrial cancer progression by modulating EMT.³⁴ H19 might function as competing endogenous RNA for miR-29b-3p to regulate EMT and metastasis of bladder cancer.³⁵ Similarly, our data showed that down-regulation of H19 suppressed EMT by increasing the expression of epithelial marker E-cadherin and decreasing the expression of mesenchymal marker Vimentin and ZEB1, which was a transcriptional repressor of E-cadherin expression in TMZ-treated glioma cells. Moreover, inducing EMT by TGF- β 1 treatment led to increased IC₅₀ values for TMZ and decreased cell apoptosis rates compared with TMZ+H19 shRNA group, suggesting that the induction of EMT counteracted the inhibitory effect of H19 shRNA on chemoresistance of glioma cells to TMZ. Our results supported the conjecture that H19 shRNA decreased chemoresistance of glioma cells to TMZ by suppressing EMT.

Having assessed the mechanism of H19 in the regulation of chemoresistance to TMZ, we further investigated the potential signal pathway involved in this mechanism. A previous study reported that H19 increased bladder cancer metastasis by associating with EZH2 and inhibiting E-cadherin expression via the Wnt/ β -Catenin pathway.²⁷ Another study reported that H19 modulated Wnt/ β -Catenin pathway by acting as a competing endogenous RNA to promote osteoblast differentiation.³⁶ Our data are also in line with previous studies which showed that H19 played a crucial role in regulating the Wnt/ β -Catenin pathway. Silencing of H19 down-regulated the expression of β -Catenin and its downstream targets c-myc and Survivin in TMZ-treated glioma cells. Activation of Wnt/ β -Catenin pathway by LiCl treatment increased EMT and chemoresistance to TMZ compared with

TMZ+H19 shRNA group, suggesting that H19 decreased chemoresistance of glioma cells to TMZ by suppressing EMT via the inhibition of Wnt/ β -Catenin pathway.

In conclusion, our present study reports that the expression of H19 was up-regulated in TMZ-resistant glioma cells and H19 decreased chemoresistance of glioma cells to TMZ by suppressing EMT via the Wnt/ β -Catenin pathway. Our study presents necessary insights to better understand the potential target of H19 in glioma in the future. However, there were still some limitations in our study. For instance: we used unspecific SYBR Green based Real-Time analysis instead of the TaqMan gene expression assay, and Western blot instead of ELISA. Moreover, our results are based on the experiments from just one cell line. It is better to use one more primary culture line to repeat the experiments. However, our present study laid the foundation for studying chemoresistance in glioma cells and we will carry out more experiments for further investigations.

Disclosure

The authors report no conflicts of interest in this work.

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